Potential of Detection and Identification of Nymphal Parasitoids (Hymenoptera: Braconidae) of *Lygus* Bugs (Heteroptera: Miridae) by Using Polymerase Chain Reaction and ITS2 Sequence Analysis Techniques

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ABSTRACT Ribosomal ITS2 DNA fragments were sequenced from four *Peristenus* species, two *Leiophron* species, and two *Lygus* species. Specific primers for polymerase chain reaction (PCR) amplification were designed from ITS2 DNA sequences to separate each species from the others. Using this molecular approach, we were able to determine whether *Lygus hesperus* Knight and *Lygus lineolaris* (Palisot de Beauvois) were parasitized by *Peristenus* and *Leiophron* parasitoids. The PCR technique was very sensitive and could detect *Peristenus stygicus* Loan DNA at a concentration of 0.01 pg/μl or 7.5×10^{-7} wasp DNA equivalents. Detection of *P. stygicus* eggs confirmed that early detection of parasitoids was possible. Parasitoid DNA was readily recovered from all *L. hesperus* nymphs that were parasitized by a single *P. stygicus* after 1 h of contact between the parasitoid and putative hosts. This study demonstrates the effectiveness of a molecular technique for detecting parasitoids developing inside their hosts.

KEY WORDS nymphal parasitoid, *Peristenus*, *Leiophron*, *Lygus* bug

TARNISHED PLANT BUG, *Lygus lineolaris* (Palisot de Beauvois), and the western tarnished plant bug, *Lygus hesperus* Knight, have a wide host range, including cotton, *Gossypium hirsutum* L. Their economic importance has become prominent in areas where lepidopterans are effectively suppressed by widespread use of cotton genetically modified to produce *Bacillus thuringiensis* (Bt) protein (Hardee et al. 2001). *Lygus* plant bugs are controlled in cotton almost exclusively with insecticides; however, resistance to pyrethroids and organophosphates has been found in many tarnished plant bug populations in the mid-south (Snodgrass 1996, Snodgrass and Scott 2000). Increased environmental concerns, coupled with the growing prevalence of insecticide resistance and outbreaks of secondary pests, have led to an increased interest in biological control (Ruberson and Williams 2000).

Several parasitic Hymenoptera attack the nymphal stage of *Lygus* bugs (Day 1999, Day et al. 1999). The species investigated in this study include *Peristenus stygicus* Loan, *Peristenus howardi* Shaw, *Peristenus pallipes* (Curtis), *Peristenus pseudopallipes* Loan, *Leiophron uniformis* (Gahan), and *Leiophron argentinensis* Shaw. *P. stygicus* was originally discovered in France and Turkey and was later released in Arizona, California, Mississippi, Texas, and Canada during the early 1970s (Coulson 1987). *P. howardi* is a recently discovered native species distributed in western North America, including Idaho, Oregon, Washington, and southwestern Canada (Day et al. 1999). *P. pallipes* and *P. pseudopallipes* are closely related univoltine species that differ in the synchronization of their phenologies (Loan 1965, 1974, 1980). Both species were previously believed to be native, but recent evidence suggests that *P. pallipes* may represent an accidental introduction to North America from Europe (Day 1999, 2002). *L. uniformis* is a native species, widely distributed throughout North America (Marsh 1979). *L. argentinensis* was recently discovered in South America (Williams et al. 2003).

P. howardi has shown potential for suppression of *L. lineolaris* in laboratory tests (Day et al. 1999). *P. stygicus* larvae outcompete other euphorine parasitoid species in *L. lineolaris* nymphs (LaChance et al. 2001). Parasitization of *L. hesperus* by *L. uniformis* can reach high levels (≈60%) (Graham et al. 1986), whereas parasitism of *L. lineolaris* by *L. uniformis* never ex-

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ceeded 5%, perhaps due to successful host encapsulation of parasitoid eggs and oviposition preference of adult wasps than for other *Lygus* species (Debolt 1989). In the northeastern United States the preferred host of *L. uniformis* is *Halticus bractatus* (Say) (≈50% parasitism); parasitism of *L. lineolaris* is incidental (Day and Saunders 1990).

Accurate assessment of the cause of host mortality is critical for establishing host–parasitoid associations. Day (1994) reviewed the two methods most frequently used: dissection of hosts and rearing of adult parasitoids from hosts. He found that depending on the specific objectives of the study, each method has advantages and disadvantages. Rearing permits the identification of parasitoids and other mortality factors, provides information on diapause of the host–parasitoid, and usually requires less technical expertise than dissection. However, dissection provides more accurate mortality data, avoids most of the confounding mortality inherent in rearing, and yields more rapid results. In a comparison of the two methods, parasitism of plant bug nymphs was 44% higher when assessed by dissection than by rearing (Day 1994). Subsequent studies of plant bug biological control have used both methods concurrently to take advantage of their unique attributes (Day 1996, 1999; Day et al. 1999).

More recently, molecular methods have been used to measure parasitism. Tilmon et al. (2000) developed a polymerase chain reaction (PCR) method for monitoring differential parasitization of *L. lineolaris* nymphs by three *Peristenus* species. This method was a two-step process that required PCR amplification followed by restriction digestion. Parasitism rates measured by the PCR method were consistent with those obtained by dissection. This study was based on prior identification of adult wasps so that the molecular characteristics unique to each species could be determined and subsequently used to distinguish between the species when in the larval stage. The work of Tilmon et al. (2000) marked an important advance in biological control of plant bugs because it demonstrated that molecular methods could be used to detect and identify parasitoids in the immature stages, thus combining important attributes of dissection (early and accurate detection) and rearing (identification of parasitoids). Zhu and Williams (2002) developed PCR methods for detection of egg parasitoid DNA at extremely low levels. They showed that specificity of detection can be achieved by careful design of primers specific to the subject organism (Zhu and Williams 2002). The PCR technique may be more effective than dissection when it is necessary to detect trace DNA of dead parasitoids within dead, moribund, or healthy hosts and therefore provide a more accurate measure of parasitism. Thus, molecular methods have great potential for studies of host–parasitoid associations, but the usefulness of this approach hinges on authoritative determination of the parasitoid species being studied. This, in turn, necessitates rearing of adult parasitoids from hosts.

Our objectives were to develop a simple one-step molecular technique, by using specific primers, for detection of and differentiation between immature stages of *Peristenus* and *Leiophron* species. The technique also was used in a preliminary study to assess parasitism of field-collected *L. lineolaris* nymphs.

Materials and Methods

***Lygus* Bugs and Parasitoids.** *L. lineolaris* nymphs were collected from goldenrod, *Solidago altissima* L., and giant ragweed, *Ambrosia trifida* L., adjacent to cotton fields ≈6 km west of Stoneville (Washington County), MS. *L. hesperus* nymphs were from a laboratory culture at the Biological Control and Mass Rearing Research Unit (BCMRRU), USDA–ARS, Mississippi State, MS. Cultures were reared on artificial diet, after Cohen (2000), and maintained in an environmentally controlled room (27°C, 50–60% RH, and a photoperiod of 16:8 [L:D] h).

P. stygicus adults were obtained from the Beneficial Insects Introduction Research Laboratory, USDA–ARS, Newark, DE, and maintained in culture in growth chambers (26.5°C, 60% RH, and a photoperiod of 16:8 [L:D] h) at the BCMRRU. Adults were held in mixed cages for 1–2 d to ensure mating and then male/female pairs were placed into separate cages containing ≈50 early instars of *L. hesperus* for parasitization. The resulting adult progeny were used in experiments.

Our collections of *P. howardi* were made in northern Idaho (Latah Co.) ≈330 km north of the location in southern Idaho from which the species was described (Day et al. 1999). In southern Idaho, *P. howardi* is seemingly multivoltine, but the insects we collected were univoltine. Apparent differences in life cycle and sex ratio between *P. howardi* collected at the two locations suggest that they may be two species, but additional research is needed to clarify this point (W. H. Day, personal communication). Therefore, we refer to the parasitoids from northern Idaho as "*P. howardi*?." *P. howardi*? was reared from *L. hesperus*, field-collected in northern Idaho (Latah Co.). Nymphs were swept from alfalfa, *Medicago sativa* L., and dog-fennel, *Eupatorium* sp., and placed in rearing chambers (Williams et al. 2003). The chambers were monitored daily and cocoons were placed individually in plastic vials (9 dram) (25 ± 1°C, 50–80% RH, and a photoperiod of 14:10 [L:D] h). After emergence, wasps were held for several days with a 10% sucrose solution, after which they were killed and preserved in 70% ethanol.

P. pallipes and *P. pseudopallipes* were obtained from the Beneficial Insects Introduction Research Laboratory. *P. pallipes* cocoons were reared (Day 1999) from *Leptopterna dolabrata* (L.) nymphs swept from alfalfa–grass fields near Blairstown, NJ, in May 2002. *P. pseudopallipes* cocoons were reared (Day 1999) from *L. lineolaris* nymphs swept from *Conyza* sp. and *Erigeron* sp. in northwestern Pennsylvania in July–August 2001. Diapausing cocoons of each species overwintered and emerged the following year (*P. pallipes* in

March 2003 and *P. pseudopallipes* in April 2002). After emergence, wasps were held for several days with a 10% sucrose solution, after which they were killed and preserved in 70% ethanol.

L. argentinensis was reared from mirid hosts collected in the field in Argentina. Nymphs were swept from wild host plants (mostly Compositae) and placed in rearing chambers (Williams et al. 2003). The chambers were monitored daily, and cocoons were placed individually in plastic vials (9 dram) and held on a laboratory bench near a window at ambient temperature (≈25°C). After emergence, wasps were held for several days with a 10% sucrose solution, after which they were killed and preserved in 100% ethanol.

L. uniformis was obtained from a laboratory colony reared on *L. hesperus* (Debolt 1981). The colony originated from field collections of *L. hesperus* nymphs from alfalfa fields in Arizona. After emergence, wasps were held for several days with a 10% sucrose solution, after which they were killed and preserved in 100% ethanol.

Isolation of Genomic DNA. Methods for DNA extraction were similar to those of Sambrook et al. (1989). Adult parasitoids or plant bugs were placed individually in 0.5-ml microcentrifuge tubes and homogenized using a motorized homogenizer (Glas-Col, Terre Haute, IN) in 100 μl of isolation buffer (0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl [pH 9.1], 0.05 M EDTA, and 1% SDS). The homogenate was spun briefly and incubated for 40 min at 65°C. Twenty microliters of 8 M potassium acetate was added to the tube and mixed by tapping. The tube containing homogenate was incubated on ice for 30 min and centrifuged for 15 min at 10,000 × *g*, after which the supernatant was transferred to a new tube. DNA was precipitated overnight at −20°C, pelleted by centrifugation, and washed with 400 μl of 70% ethanol. DNA pellets were air-dried and resuspended in 100 μl of distilled water.

PCR Amplification, Cloning, and Sequencing of ITS2. The DNA fragment was amplified using the forward primer 58SF (5′-TGTGAACTGCAGGACACATGAAC-3′) and reverse primer ITS2R (5′-AGTCTCGCCTGCTCTGAGGT-3′) of Porter and Collins (1991). PCR products were separated on a 0.8% low melting point agarose gel. DNA fragments were excised from the gel and extracted using a Wizard PCR Preps DNA purification system (Promega, Madison, WI). The isolated DNA fragments were cloned into a pGEM-T vector (Promega) at 22°C for 1 h, and then at 4°C overnight. Transformed *Escherichia coli* cells with vectors were plated on LB/ampicillin/IPTG/X-Gal medium. White colonies were subjected to PCR amplification to confirm the presence of inserts and the expected sizes of fragments. PCR-confirmed colonies were inoculated into 4 ml of LB/ampicillin cultures and plasmid DNA was extracted using a Plasmid Mini kit (QIAGEN, Santa Clarita, CA). DNA inserts were sequenced using an automated sequencer (ABI Prism 3700, Amersham Biosciences Inc., Piscataway, NJ). The ribosomal ITS2 DNA sequence was confirmed by homology searching of GenBank of the National Center for Biotechnology Information by using the Blastn protocol (Altschul et al. 1997).

Primer Design and PCR Amplification of Individual Wasp DNA. After ITS2 DNA sequences were obtained from parasitoids and plant bugs, the BioEdit (version 5.09, by Tom Hall, Ibis Therapeutics, Carlsbad, CA) program was used to conduct multiple sequence alignment and phylogenetic analyses. Sequence identity was determined using CLUSTALW pairwise alignment protocol (Thompson et al. 1994) with gap opening penalty of 10, gap extension penalty of 0.1 (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalwan.html). Oligo Analyzer 2.5 (http://www.idtdna.com/) was used to design primers for separating each parasitoid species from two host plant bug species and from the other three parasitoid species. Common primers for the two *Peristenus* species and the two *Leiophron* species were also designed. Four pairs of specific primers were designed to separate each species from the others. PCR reactions (25 μl) contained 10 mM Tris-HCl at pH 9, 1.5 mM $MgCl_2$, 0.5 μM of each primer, 50 mM KCl, 0.1 mM of each dNTP, 0.05 U/μl of *Taq*DNA polymerase (Promega), and 2.5 μl of DNA template (≈15 ng) and were performed in a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA). DNA was initially denatured for 3 min at 94°C, and the PCR amplification was conducted for 45 cycles, with 30-s denaturing at 94°C, 30-s annealing at 60°C, and 1-min extension at 72°C. PCR products (5–8 μl) were separated on 1% agarose gel containing 0.5 μg/ml ethidium bromide and photographed under UV light.

Detection Specificity. Six pairs of primers were tested for their specificity. DNA was individually extracted from five adults of each of four wasp species and five nymphs of *L. hesperus.* Parasitoid and *L. hesperus* DNAs were amplified with all six pairs of primers to produce the expected DNA fragment size from the corresponding DNA template. To differentiate *P. howardi*?, *P. pallipes,* and *P. pseudopallipes,* PCR amplification with single 10-mer primer was carried out by screening 30 10-mer primers (Operon Technologies, Inc, Alameda, CA). PCR reaction contained 2.5% dimethyl sulfoxide. DNA concentration and annealing temperature were optimized to ≈6 ng/30 μl and 37°C, respectively, to obtain reproducible band patterns. PCR amplifications were repeated with primers generating variable band patterns among the three species.

Detection Sensitivity. Detection sensitivity was determined by adding serial concentrations of the DNA template to each PCR reaction to estimate the lowest concentration needed to produce a visible fragment band on agarose gel. Primers PsF2 and PsR2 were selected to amplify *P. stygicus* DNA, which was extracted from adults preserved in ethanol for 5 mo. DNA was diluted to 11 concentrations, including 200, 100, 20, 10, 2, 1, 0.2, 0.1, 0.02, 0.01, 0.002 pg of DNA per microliter PCR reaction. These concentrations were converted to 1.5×10^{-2}, 7.5×10^{-3}, 1.5×10^{-3}, $7.5 \times$

10^{-4}, 1.5×10^{-4}, 7.5×10^{-5}, 1.5×10^{-5}, 7.5×10^{-6}, 1.5×10^{-6}, 7.5×10^{-7}, and 1.5×10^{-7} DNA equivalents of an adult parasitoid (one adult DNA is 460 ± 80 ng). Detection sensitivity was also determined using LuF1 and LuR1 primers to amplify *L. uniformis* DNA extracted from a wasp preserved in ethanol for 2 yr. DNA was diluted to 10 different concentrations equal to 2.7×10^{-3}, 1.3×10^{-3}, 2.7×10^{-4}, 1.3×10^{-4}, 2.7×10^{-5}, 1.3×10^{-5}, 2.7×10^{-6}, 1.3×10^{-6}, 2.7×10^{-7}, and 1.3×10^{-7} DNA equivalents of an adult wasp. PCR amplification was performed for 45 cycles, and the PCR product was subjected to gel electrophoresis.

Detection of *P. stygicus* Egg Stage. This experiment determined whether PCR amplification could be used to detect *P. stygicus* eggs developing within host nymphs. A single female adult was released into a series of glass test tubes each containing a single ≈ 3-d-old (first instar) *L. hesperus.* After stinging (which usually occurred within 20 s), the wasp was immediately transferred to another tube. In total, 11 nymphs were stung in this manner, all within 2 h. Putatively parasitized nymphs remained within individual test tubes and were kept at ambient conditions for ≈20 h. The nymphs were then frozen (−20°C) and preserved in 100% ethanol. DNA was isolated from *L. hesperus* nymphs and subjected to PCR amplification for 45 cycles with an annealing temperature of 58°C. Primers PsF3 and PsR2 were used in the PCR amplification.

Results and Discussion

PCR and ITS2 Cloning. With the use of primers 58SF and ITS2R, the PCR amplification generated a distinct band from each parasitoid species and from *L. hesperus.* The size of the ITS2 fragment for six parasitoids was ≈750 nucleotide base pairs and the ITS2 fragment for *L. hesperus* was ≈1,300 bp. All these ITS2 DNA fragments were successfully cloned and sequenced. Sequences were confirmed as ITS2 by Blastn similarity searching the GenBank database (Altschul et al. 1997). Five ITS2 fragments were sequenced from individual wasp of *P. stygicus* (768.6 bp), *P. howardi*? (773.8 bp), *P. pallipess* (772 bp), and *P. pseudopallipes* (773.5 bp), and four ITS2 fragments were sequenced from individual wasp of *L. argentinensis* (703.8 bp) and *L. uniformis* (715.6 bp). Results showed that the ITS2 DNA fragments were similar in length within a taxonomic group. The ITS2 DNA fragments of the two *Leiophron* species (*L. argentinensis* and *L. uniformis*) were slightly shorter than those of the two *Peristenus* species. The ITS2 fragment of *L. hesperus* was much longer than those of all parasitoids. It contained 1,299 nucleotides, which was ≈450 bp longer than the fragments of any of the parasitoids. However, the ITS2 fragment length of *L. hesperus* was approximately the same length of the ITS2 fragment of *L. lineolaris,* which contained 1,302 nucleotides (Zhu and Williams 2002).

The primers 58SF and ITS2R flank to regions of the 5.8S and the 28S ribosomal DNA, respectively. These sequences are highly conserved, and the corresponding primers have worked for many insects (Wesson et al. 1992, van Kan et al. 1996). By using these primers, PCR amplification of the unparasitized plant bug DNA generated an ≈1,300-bp fragment. If the plant bug had been parasitized, two widely separate bands (≈750 and ≈1,300 bp) would show. To recognize particular parasitoid species, specific primers must be designed from the rDNA sequence of concerned species.

Table 1. Phylogenetic distances among six parasitoid species

	Ps	Ph	Pp	Ppp	La	Lu
Ps	0.038	0.1725	0.1701	0.1704	0.3317	0.3502
Ph		0.0384	0.0649	0.0728	0.3119	0.3229
Pp			0.0471	0.0701	0.3167	0.3323
Ppp				0.0647	0.3183	0.3356
La					0.0517	0.102
Lu						0.0262

La, *L. argentinensis;* Lu, *L. uniformis;* Ph, *P. howardi*?; Pp, *P. pallipes;* Ppp, *P. pseudopallipes;* Ps, *P. stygicus.*

ITS2 Variation and Primer Design. ITS2 fragments between the 5.8S and 28S ribosomal DNAs are good candidates for markers. They have highly repetitive and relatively divergent sequences and have proven useful for comparison between closely related insect species and subspecies (Black et al. 1989, Collins et al. 1990, Porter and Collins 1991, Chen et al. 1992, Stouthamer et al. 1999).

We sequenced the ITS2 DNA fragments of all six parasitoids and one host species (*L. hesperus*). The ITS2 sequences from *L. lineolaris* (Zhu and Williams 2002) also was used in multiple alignment. Due to length and sequence variation between parasitoid and *L. hesperus* ITS2 DNAs, multiple alignment introduced many gaps. Thus, only the parasitoid ITS2 sequences were used for alignment and designing of primers. Total 28 ITS2 sequences from six parasitoid species were aligned, and the phylogenetic distances among six species were calculated (Table 1; Fig. 1). Phylogenetic analyses indicated that intraspecific variations exist. Multiple sequence alignment resulted in 90.3, 89.33, 87.87, 83.68, 85.15, and 90.52% sequence identity for *P. stygicus, P. howardi*?, *P. pallipes, P. pseudopallipes, L. argentinensis,* and *L. uniformis,* respectively. However, the pairwise sequence comparison revealed that sequence identity reached 94.89 ± 0.76, 94.99 ± 0.67, 93.49 ± 0.54, 91.36 ± 0.58, 92.90 ± 0.96, and 95.75 ± 0.53% for each of above-mentioned species. Interspecific variations were relatively great among *P. stygicus, P. howardi*?, *L. argentinensis,* and *L. uniformis,* and the sequences were clearly separated into distinct groups. But the variations were low among *P. howardi*?, *P. pallipes, P. pseudopallipes,* and their ITS2 sequences were located in one phylogenetic branch (Table 1; Fig. 1).

Several pairs of primers were designed using Oligo Analyzer (Table 2). Primers PsF1 and PsR2 were common to all four *Peristenus* species, and primers PsF1 and LR1 were common to both *Leiophron* species. The other four pairs of primers were specific to the corresponding parasitoid species. Primer sequences and expected band sizes are given in Table 2. No critical

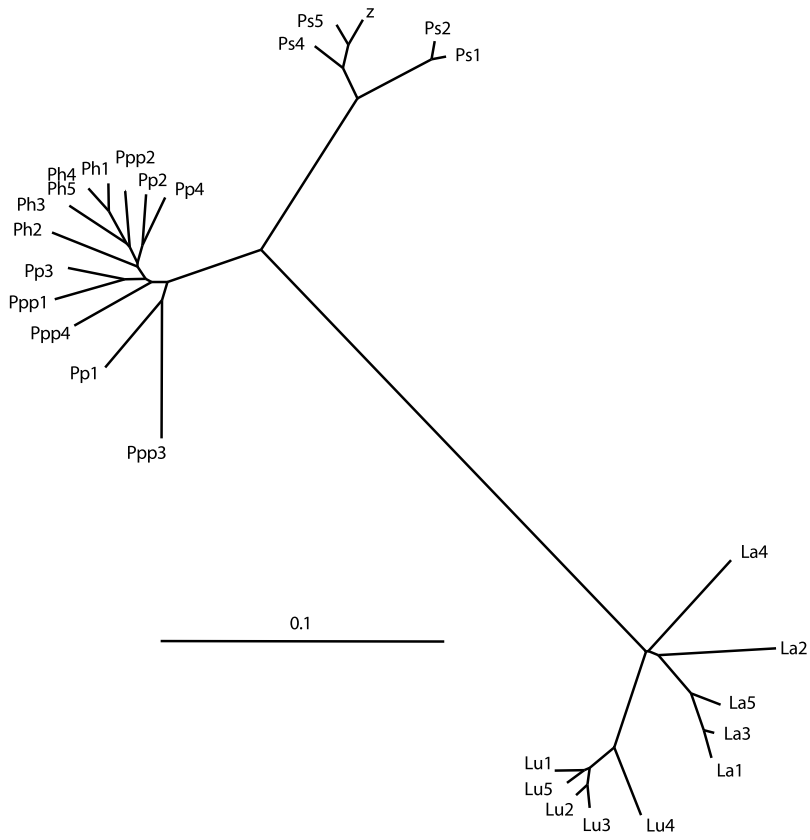

Fig. 1. Phylogenetic analysis of 28 ITS2 rDNA sequences by using Fitch Phylogenetic Tree method (BioEdit). Ps, *P. stygicus*; Ph, *P. howardi*?; Pp, *P. pallipes*; Ppp, *P. pseudopallipes*; La, *L. argentinensis*; Lu, *L. uniformis*.

mismatch (Kwok et al. 1990) between primers and variable targets (due to intraspecific variation) was found in all primers.

Detection Specificity. Each pair of primers was tested with all four parasitoid DNAs and *L. hesperus* DNA. The ITS2 DNA of *L. hesperus* shared 95.62% sequence identity with the ITS2 DNA of *L. lineolaris* and was used to represent both *Lygus* species in the PCR specificity study.

Primers PsF1 and PsR2 were designed from *Ps* sequence, and each primer had one nucleotide difference from corresponding positions of the *Ph* sequences. However, this mismatch did not significantly affect PCR yield, and PsF1 + PsR2 could be used to

Table 2. Primer sequences for specific detection of the nymph parasitoids of the plant bug

Primer name	Sequence	Target species	Band size (bp)
58SF	TGTGAACTGCAGGACACATGAAC	*P. stygicus*	767
ITS2R	AGTCTCGCCTGCTCTGAGGT	*P. howardi*?	773
		L. argentinensis	704
		L. uniformis	718
		L. lineolaris	1,302
		L. hesperus	1,299
PsF1	ACCTGGCTGAGGGTCGTTTA	*P. stygicus*	455
PsR2	CGACATCTGTCCTTCTGTACTTC	*P. howardi*?	456
PsF3	TGTGAATTTATTCATGTAACCAAAGCT		
PsR2	CGACATCTGTCCTTCTGTACTTC	*P. stygicus*	264
PhF1	CAATAAATTATTTATCTGGCCAAACTC		
PhR1	ATACCATGATTGTTGAACATAAATGC	*P. howardi*?	432
PsF1	ACCTGGCTGAGGGTCGTTTA	*L. argentinensis*	477
LR1	CGGTGTAAACAATCATTATAGACCATTG	*L. uniformis*	485
LaF1	CTAAGTACTGATTGCATCGTTGATG		
LaR1	GTCAATTGTAATTGCCATTGAGTGGA	*L. argentinensis*	445
LuF1	GAATCGTTGATATTGAGTGAGTATAT		
LuR1	ATCAAGAAAGGAATGTGTCACAGGT	*L. uniformis*	469

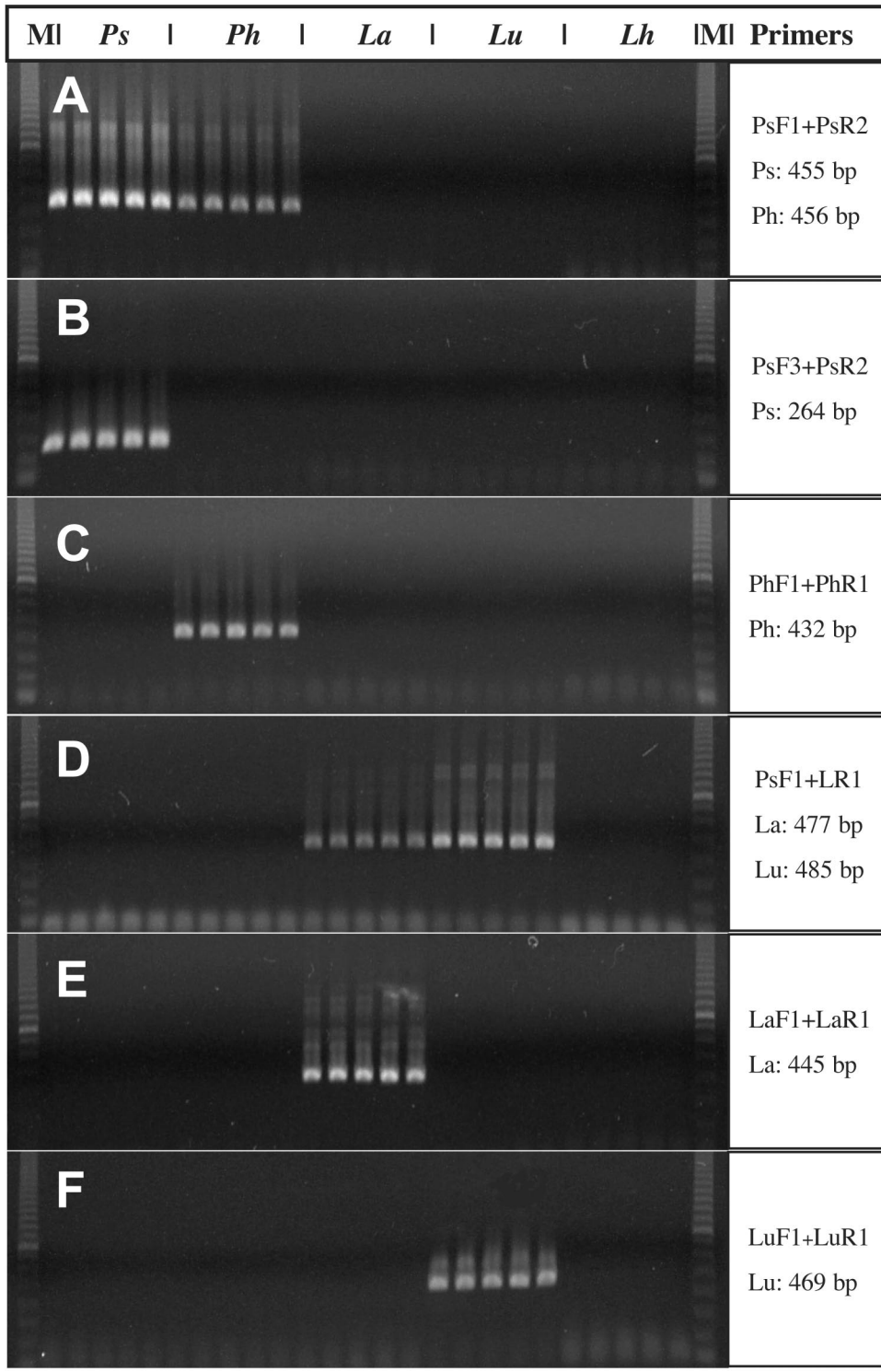

Fig. 2. Specificity of primers. (A) PCR amplification of *P. stygicus* (*Ps*), *P. howardi*? (*Ph*), *L. argentinensis* (*La*), *L. uniformis* (*Lu*), *and L. hesperus* (*Lh*) DNAs with PsF1 + PsR2 primers. (B) PCR amplification with PsF3 + PsR2 primers. (C) PCR amplification with PhF1 + PhR1 primers. (D) PCR amplification with PsF1 + LR2 primers. (E) PCR amplification with LaF1 + LaR1 primers. (F) PCR amplification with LuF1 + LuR1 primers. M, 100-bp DNA marker (Pharmacia, Peapack, NJ).

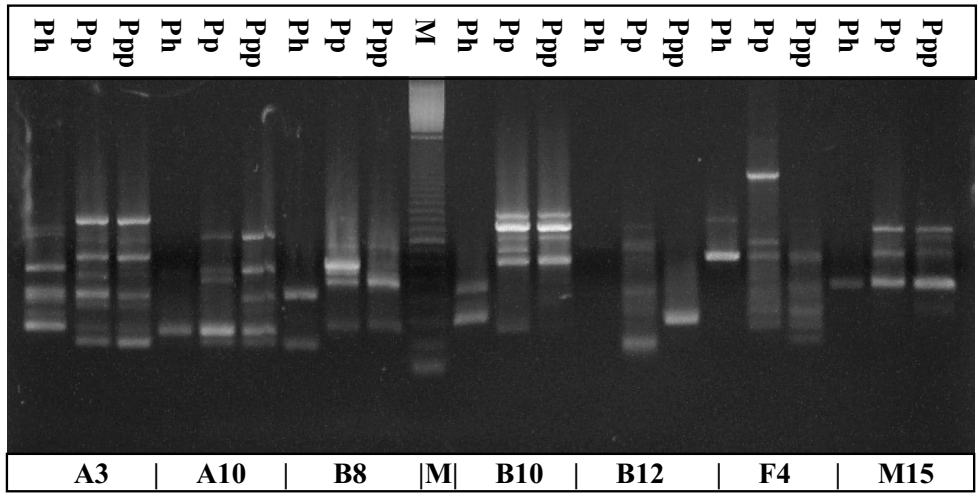

Fig. 3. Molecular differentiation of three closely related *Peristenus* species: Ph, *P. howardi*?; Pp, *P. pallipes*; and Ppp, *P. pseudopallipes* by using seven oligo primers. Primer sequences are A3, AGTCAGCCAC; A10, GTGATCGCAG; B8, GTCCACACGG; B10, CTGCTGGGAC; B12, CCTTGACGCA; F4, GGTGATCAGG; and M15, GACCTACCAC. M, 100-bp DNA marker (Pharmacia).

detect all selected *Peristenus* spp. PCR amplification with these two primers generated a 455-bp fragment and a 456-bp fragment from *Ps* and *Ph* DNA, respectively. No DNA fragment was amplified from any of *La*, *Lu*, or *Lh* DNA (Fig. 2A). PsF1 also matches to the corresponding positions of *La* and *Lu* DNAs. PCR amplification became specific to two *Leiophron* species DNA templates once a reverse primer, LR1, was included with forward primer PsF1. A 477- and a 485-bp ITS2 DNA fragment was amplified from *La* and *Lu*, respectively. No DNA fragment was generated from *Ps*, *Ph*, or *Lh* DNAs (Fig. 2D). Other four pairs of primers, PsF3 + PsR2, PhF1 + PhR1, LaF1 + LaR1, and LuF1 + LuR1 exhibited high specificity, and 264-, 432-, 445-, and 469-bp fragments were amplified from corresponding DNA templates *Ps*, *Ph*, *La*, and *Lu*, respectively. No cross-amplification was obtained from nontargeted parasitoids or *Lh* DNAs (Fig. 2, B, C, E, and F).

P. howardi?, *P. pallipes*, and *P. pseudopallipes* had similar ITS2 sequences (average distance of ≈0.0692; Table 1). The primers designed for *P. howardi*? also work for *P. pallipes* and *P. pseudopallipes* DNAs. We aligned all ITS2 sequences from all three species and found these three species shared all variable nucleotide positions that were variable within each species. We also examined 18S rDNA region (data not shown) and found that it was hard to separate these three species using PCR primers flanking to rDNA. To differentiate these three species, 10-mer short oligo primers were used to explore genetic variations among *P. howardi*?, *P. pallipes*, and *P. pseudopallipes.* Results (Fig. 3) demonstrated that primers A3, A10, B8, B10, B12, F4, and M15 were able to generate unique band patterns to separate *P. howardi*? from *P. pallipes* and *P. pseudopallipes. P. pallipes* and *P. pseudopallipes* exhibited similar band patterns for primers A3, B10, and M15, but the other primers, A10, B8, B12, and F4 could produce specific band patterns to separate *P. pallipes* from *P. pseudopallipes* and *P. howardi*? (Fig. 3).

Detection Sensitivity. The sensitivity test determined the quantity of parasitoid DNA necessary to generate a visible band on agarose gel. Two wasps and two pairs of primers were used in this study. ***Ps*** DNA was isolated from adult wasps preserved in 100% ethanol for 5 mo. Eleven concentrations were prepared from ***Ps*** DNA. PCR amplification with PsF2 and PsR2 primers produced a 388 bp fragment. Band intensity remained high for those DNA concentrations above 7.5×10^{-4} parasitoid DNA equivalents (Fig. 4A). Band intensity decreased as DNA concentration decreased below 1.5×10^{-4}, but maintained visible intensity until the DNA concentration decreased to 7.5×10^{-7}. Bands disappeared when DNA concentration fell below 1.5×10^{-7} (Fig. 4A). This result indicated that detectable *Ps* DNA concentration was 0.01 pg/μl PCR reaction and that the PCR system was very sensitive for detection of trace amounts of parasitoid DNA within hosts. Results in Fig. 4B and C show that detectable *Lu* DNA levels could be reduced to 1.3×10^{-5} wasp DNA equivalents or 10 pg/μl PCR reaction to generate a 469-bp band. PCR sensitivity to *Lu* DNA was reduced, in comparison with *Ps* DNA, possibly due to a 2-yr period of storage in ethanol. Result from Fig. 4C also showed that *Lh* DNA had no negative effect on PCR detection of *Lu* DNA. The band intensity generated from *Lu* DNA was similar to that from mixtures of *Lu* and *Lh* DNAs.

Detection of *P. stygicus* Egg Stage. Because the PCR system was highly sensitive, as little as 1 pg of DNA was needed to generate a visible band. This technique facilitated early detection of the parasitic wasps within their hosts. Carignan et al. (1995) reported that the egg stage of *P. digoneutis* in *L. lineolaris* nymphs was

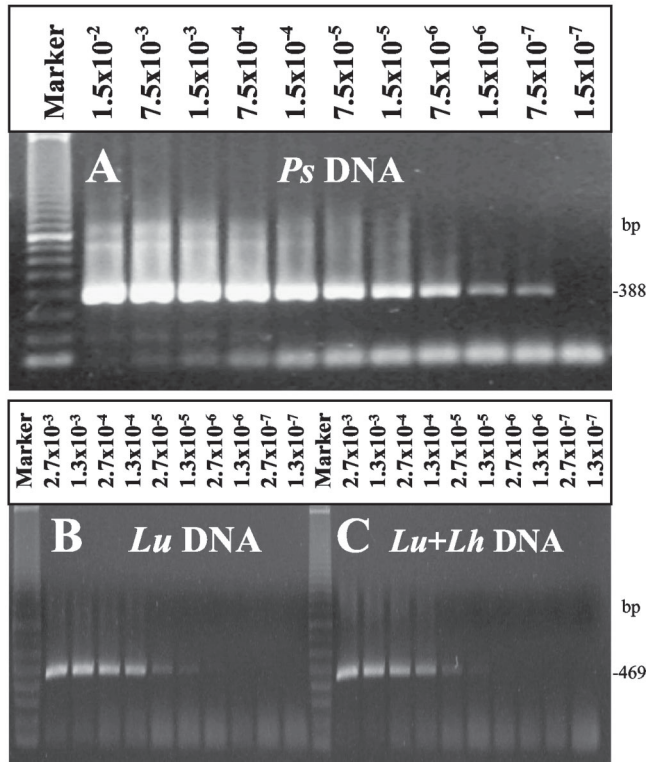

Fig. 4. Sensitivity of PCR amplification. (A) PCR amplification of 11 concentrations of *P. stygicus* (*Ps*) DNA with PsF2 + PsR2 primers. (B) PCR amplification of 10 concentrations of *L. uniformis* (*Lu*) DNA with LuF1 + LuR1 primers. (C) PCR amplification of 10 concentrations of *L. uniformis* (*Lu*) DNA mixed with constant amount of *L. hesperus* (*Lh*) DNA with LuF1 + LuR1 primers.

5 d at ≈21°C, and it is likely that a similar condition exists for *P. stygicus* eggs in *L. hesperus* nymphs. PCR amplification of the *L. hesperus* DNA with PsF3 + PsR2 primers generated an expected 264-bp band from all nymphal DNAs (Fig. 5). Our results suggest that this technique can greatly shorten the time period for confirming that parasitization had occurred; from ≈1 wk to <2 d.

In conclusion, this study demonstrates the advantages and limitations of PCR techniques for identification of immature stages of nymphal parasitoids of *Lygus* bugs. An important advantage is the ability to assess parasitism sooner than would be possible by rearing and to determine the identity of immature parasitoids, which is very difficult by dissection (Carignan et al. 1995). The PCR technique also provides a more accurate evaluation of parasitism than rearing, because disproportional mortality of parasitized hosts during the rearing process would be minimized. Another important advantage of the PCR technique is the ability to detect parasitoids in the egg stage, thus facilitating assessment of parasitism (and identification of parasitoids) earlier than possible with dissection or rearing. Molecular studies of braconid systematics also will benefit from the PCR technique. Limitations of the PCR technique include the relatively high costs of supplies and equipment and having personnel with appropriate technical expertise. Using this technique, the time required for assessment of parasitism is slightly longer than for dissection, but much less than for rearing. It is important to understand that there is no "best" method for detection of

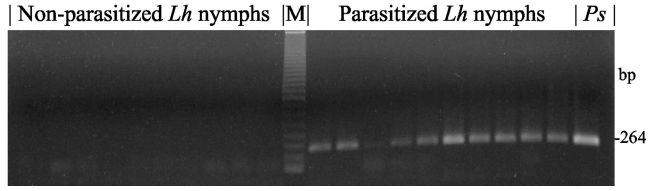

Fig. 5. Detecting parasitic wasps within host insects by PCR amplification of nonparasitized and parasitized *L. hesperus* (*Lh*) nymph DNA and *P. stygicus* (*Ps*) DNA with PsF3 + PsR2 primers. M, 100-bp DNA marker (Pharmacia).

parasitism of *Lygus* nymphs; rearing, dissection, and molecular methods all have attributes and limitations. However, the PCR technique provides a powerful new tool that when used in concert with the dissection and rearing methods, will facilitate more rigorous and informative investigations on parasitoid–*Lygus* interactions than previously possible. Collaborations between research laboratories with different areas of expertise would expedite such work.

DNA Sequence Registration

DNA sequences have been deposited in GenBank with accession number AY170220 and AY590800-AY590803 for *P. stygicus*, AY170221 and AY590804-AY590807 for *P. howardi*?, AY170222 and AY590814-AY590817 for *L. argentinensis*, AY170223 and AY590818-AY590821 for *L. uniformis*, AY519659 and AY590808-AY590810 for *P. pallipes*, and AY519660 and AY590811-AY590813 for *P. pseudopallipes.*

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